J Surg Med. 2020;4(12):1101-1103.
DOI: 10.28982/josam.816697

Research article
Araştırma makalesi

Journal of Surgery and Medicine
e-ISSN=2602-2079

Melatonin prevents post-traumatic ischemic damage in rats

Melatonin ratlarda post-travmatik iskemik beyin hasarını önlemektedir

Cengiz Gölçek [1], Mehmet Arif Aladağ [2], Harika Gözükara [3]

[1] Department of Neurosurgery, Malatya Education Research Hospital Malatya, Turkey
[2] Department of Neurosurgery, Medical Faculty, İnönü University, Malatya, Turkey
[3] Department of Biostatistics, Medical Faculty, İnönü University, Malatya, Turkey

ORCID ID of the author(s)
CG: 0000-0002-1969-9080
MAA: 0000-0003-3872-3741
HG: 0000-0003-1208-4072

Corresponding author / Sorumlu yazar:
Cengiz Gölçek
Address / Adres: Malatya Eğitim Araştırma Hastanesi, Nöroşirürji Kliniği, Malatya, Türkiye
E-mail: cengizgolcek@gmail.com

Ethics Committee Approval: This study was carried out in the experimental research laboratories of Medical Faculty of İnonu University in accordance with the Ethical Committee's guidelines on the maintenance and usage of rats (2011/A-85).
Etik Kurul Onayı: Bu çalışma, İnönü Üniversitesi Tıp Fakültesi deneysel araştırma laboratuvarlarında, Etik Kurul'un sıçanların bakımı ve kullanımına ilişkin yönergeleri çerçevesinde gerçekleştirilmiştir (2011/A-85).

Conflict of Interest: No conflict of interest was declared by the authors.
Çıkar Çatışması: Yazarlar çıkar çatışması bildirmemişlerdir.

Financial Disclosure: The authors declared that this study has received no financial support.
Finansal Destek: Yazarlar bu çalışma için finansal destek almadıklarını beyan etmişlerdir.

Published: 12/21/2020
Yayın Tarihi: 21.12.2020

Abstract

Aim: Brain trauma is among the leading causes of mortality and long-term disability in the world. Studies suggested that cerebral ischemia is an important mechanism of secondary neuronal injury in traumatic brain injury (TBI), and that melatonin has protective effects on the brain after trauma. It was also shown that melatonin alleviates the formation of cerebral ischemia and ischemic brain damage in many cerebral pathophysiological processes. However, there is no study which investigates the effects of melatonin on cerebral ischemia after brain trauma. Therefore, we aimed to induce experimental focal brain trauma in rats and assess the effects of melatonin on posttraumatic cerebral ischemia.

Methods: The animals used in this research were divided into four groups as follows: Control group (Group 1), Traumatic Brain Injury (TBI) group (Group 2), TBI plus Placebo group (Group 3), and TBI plus Melatonin group (Group 4). Brain trauma was induced using the weight drop technique in all groups except the Control group (Group 1). The groups with induced brain trauma were separated into five sub-groups to be sacrificed at the given times (12, 24, 72, 120 and 168 hours). Hematoxylin and eosin (H&E) staining was applied to count the number of red neurons, which indicate the grade of cerebral ischemia.

Results: Our results showed that the number of red neurons was significantly less ($P<0.05$) in the melatonin-treated groups compared to those in the trauma and placebo groups within the same amount of time.

Conclusion: The present study found that melatonin markedly inhibits the progression of cerebral ischemia after brain trauma. Therefore, melatonin can be used as a potential therapeutic agent to prevent posttraumatic secondary cerebral injuries. However, further studies are needed to investigate the mechanism of its effect.

Keywords: Melatonin, Traumatic brain damage, Secondary injury, Ischemic injury

Öz

Amaç: Beyin travması, dünyadaki ölümlerin ve uzun süreli sakatlığın önde gelen nedenleri arasındadır. Çalışmalar, serebral iskeminin travmatik beyin hasarında (TBH) sekonder nöronal hasarın önemli bir mekanizması olduğunu göstermiştir. Bilimsel araştırmalar, melatoninin travma sonrası beyin üzerinde koruyucu bir etkiye sahip olduğunu göstermiştir. Ayrıca melatoninin birçok serebral patofizyolojik süreçte beyin iskemik beyin hasarını hafiflettiği gösterilmiştir. Bununla birlikte, melatoninin beyin travması sonrası serebral iskeminin oluşması üzerine olan etkisini araştıran herhangi bir çalışma yoktur. Bu nedenle, sıçanlarda deneysel fokal beyin travması oluşturduk ve zamansal seyri içinde melatoninin travma sonrası serebral iskemi oluşumu üzerindeki etkisini araştırdık.

Yöntem: Hayvanlar dört gruba ayrıldı: kontrol (Grup 1), Travmatik Beyin Hasarı (TBH) (Grup 2), TBH artı plasebo (Grup 3) ve TBH artı melatonin (Grup 4). Beyin travması, ağırlık bırakma tekniği ile kontrol grubu dışındaki tüm gruplarda oluşturuldu. Beyin travması oluşturulan grupların her biri belirli zamanlarda (12, 24, 72, 120 ve 168 saatlerde) sakrifiye edilecek şekilde beş alt gruba ayrıldı. İskeminin derecesini göstermede kırmızı nöronların sayısını belirlemek için hematoksilin-eozin (H&E) boyama kullanıldı.

Bulgular: Sonuçlarımız, melatonin ile tedavi edilen gruplarda travma ve plasebo gruplarına kıyasla kırmızı nöron sayısının anlamlı bir şekilde azaldığını ($P<0.05$) gösterdi.

Sonuç: Bu çalışma, melatoninin beyin travması sonrasında oluşan iskemiyi azaltığını desteklemektedir. Bu yüzden, melatonin travma sonrası oluşan ikincil serebral yaralanmaları önlemek için potansiyel bir terapötik ajan olarak kullanılabilir. Ancak onun bu etkisinin mekanizmalarını araştırmak için daha ileri çalışmalar gerekmektedir.

Anahtar kelimeler: Melatonin, Travmatik beyin hasarı, İkincil hasar, İskemik hasar

How to cite/Atıf için: Gölçek C, Aladağ MA, Gözükara H. Melatonin prevents post-traumatic ischemic damage in rats. J Surg Med. 2020;4(12):1101-1103.

Introduction

Although it is not possible to treat post-traumatic primary cerebral damage caused by direct mechanical factors, post-traumatic secondary injuries brought about by multidimensional processes beginning after the primary injury are likely to respond to treatment [1,2].

One of the most common causes of post-traumatic secondary damage is ischemia [3-5]. Further research on this topic found that pathophysiological processes leading to posttraumatic secondary damage are similar to those leading to ischemic damage [6,7].

Melatonin, a natural agent, has a protective effect on neurons in post-traumatic injuries [8]. Melatonin can prevent the formation of both ischemia and ischemic damage after subarachnoid hemorrhage and ischemia reperfusion injury [9,10]. However, to the best of our knowledge, there is no study on the effect of melatonin on ischemia formation after brain trauma.

Due to all the above-mentioned reasons, we investigated the effect of melatonin on post-traumatic cerebral ischemia formation in experimentally induced head trauma in rats.

Materials and methods

Ethical statement

This study was carried out in the experimental research laboratories of Medical Faculty of İnonu University in accordance with the Ethical Committee's guidelines on the maintenance and usage of rats (2011/A-85-Analysis of the temporal development of secondary injury in head injuries).

Experimental procedure

The experiment was performed on eighteen, 15-week-old Wistar albino rats weighing between 200 and 250g. The animals were kept under the standard conditions of 12-hour light and dark cycles, at a 20^0C steady room temperature, with a humidity ranging from 40% to 60%. The lab rats had free access to standard dry pellets and tap water throughout the study.

The rats were divided into four groups: Group 1: Control (n=5), Group 2: Trauma (n=25), Group 3: Trauma plus Placebo (n=25), and Group 4: Trauma plus Melatonin (n=25). The rats in Group 1 were sacrificed under anesthesia and their data were considered the basal level for comparison. To determine the change with time, groups 2-4 were further divided into 5 subgroups (A-E), containing 5 rats each.

One day before the operation, the rats were starved and given Enrofloxacin (Baytril, 2.27 mg/kg, Bayer) subcutaneously for prophylactic purposes. The rats were anesthetized with ketamine hydrochloride (50mg/kg) and Xylazine (10mg/kg) for the operation. To stabilize their body temperature, they were set on operation tables pre-heated to 37℃. Anesthesia was maintained with additional ketamine injections, as needed.

The rats in the control group were sacrificed without performing any procedures. The surgical sites of the rats in trauma, trauma plus placebo and, trauma plus melatonin groups were shaved and antiseptically cleaned. A midline longitudinal scalp incision, and periosteal and muscular dissection were made to expose the surface of the skull. A craniotomy (10 mm x 15 mm), centered over the right parietal bone, was performed using a dental drill. For the induction of brain injury, we used the weight drop technique modified by Feeney et al. [11], which included a 9 g weight dropped from a height of 50 cm onto a 10 mm diameter piston resting on the exposed dura. Thus a head trauma was created by applying 450 g/cm force according to the formula of $EP=m.g.h$.

To explore changes with time, trauma, trauma plus placebo and, trauma plus melatonin groups were divided into 5 subgroups (A-E), with 5 rats in each. Each group was sacrificed at specified time points after the injury, as follows: Subgroup A: Sacrificed at the 12^{th} hour, B: Sacrificed at the 24^{th} hour, C: Sacrificed at the 72^{nd} hour, D: Sacrificed at the 120^{th} hour, and E: Sacrificed at the 168^{th} hour after injury. Six hours after procedure, 1 ml 2.5% alcohol and 20 mg/kg/day melatonin (Sigma) in 1 ml 2.5% alcohol solution were injected intraperitoneally in the trauma plus placebo and trauma plus melatonin groups respectively, while Group 2 received no medications. Intraperitoneal melatonin or alcohol injections (warmed at 37^0C) were continued until sacrifice.

Sample collection and sacrifice of rats

At the end of periods mentioned above for each subgroup, the animals were re-anesthetized, and the ascending aorta was cannulated retrogradely through a thoracotomy. The cranio-cervical circulation was perfused with 200 ml of heparinized iso-osmotic phosphate buffer saline (0.1M, pH 7.4) at a physiological mean arterial pressure (80–90mmHg) via a peristaltic pump (May=PRS9508=991129-1). The perfusion was followed by 200ml of 0.1M phosphate buffer saline containing 4% paraformaldehyde at a physiological mean arterial pressure as above. Brains were rapidly resected and right and left hemispheres were separated. The right hemispheres, which contained the contusion epicenter, were post-fixed in 4% formalin and processed for paraffin embedding. Representative sections were sliced into 5 μm thick sections and stained with hematoxylin-eosin (H&E) for the evaluation of the degree of ischemia. The number of red-stained neurons typically observed in early injuries were counted [12] in 10 different areas on a light microscope (Olympus, BX400) and added.

Statistical analysis

The data of the control group represented the basal level of all parameters. The distribution of the data was analyzed with the Shapiro-Wilk test. Data was presented as median (min-max). Kruskal Wallis H test was performed for comparison of groups. Multiple comparisons were carried out with the Conover test. A P-value <0.05 was considered statistically significant.

Results

The images of samples from Groups 2 and 4 under light microscopy are shown in Figures 1A and 1B. The distribution of pink acidophilic neurons (ischemic red neurons) by groups is shown in Table 1.

The number of red neurons in sub-groups (A-E) of Trauma (Group 2), Trauma plus Placebo (Group 3) and Trauma plus Melatonin (Group 4) groups were 10 (7-18), 15 (5-18), 15 (13-18), 16 (14-18), 16 (14-17), and 10 (8-17), 14 (5-16), 15 (14-17), 15 (14-18), 16 (15-18) and, 6 (6-7), 5 (4-5), 7 (6-8), 7 (6-8), 6 (5-8), respectively.

While the number of red neurons in Trauma and Trauma plus Placebo groups significantly increased at 12, 24, 72,

120, 168 hours, there was no significant difference between Trauma and Trauma plus Placebo groups. In all of subgroups of Trauma and Trauma plus Placebo groups, the number of red neurons were significantly higher than that of the control group. Significant elevation in the number of these neurons over time in the post-traumatic brain indicates cerebral ischemia.

Table 1: Comparison of the number of pink acidophilic dead neurons (red neurons) between the groups and subgroups (RN: Red Neuron)

Subgroup (Time) Group	A (12. S) RN(n) median (min-max)	B (24.S) RN(n) median (min-max)	C (72. S) RN(n) median (min-max)	D (120. S) RN(n) median (min-max)	E (168. S) RN(n) median (min-max)	*P*-value
1.Basal level	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	1.000
2.Trauma	10 (7-18)	15 (5-18)	15(13-18)	16(14-18)	16 (14-17)	<0.001
3.Trauma+Placebo	10 (8-17)	14(5-16)	15(14-17)	15(14-18)	16(15-18)	<0.001
4.Trauma+Melatonin	6 (6-7)	5 (4-5)	7 (6-8)	7 (6-8)	6 (5-8)	<0.001
P-value	<0.001	0.002	<0.001	<0.001	<0.001	

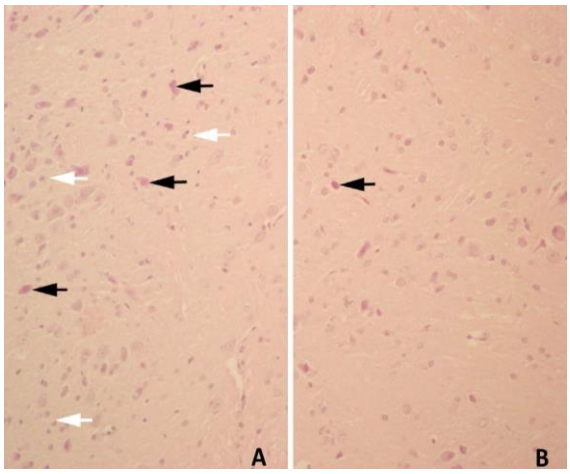

Figure 1: Pink acidophilic dead neurons "red neurons" (black arrows) and moderate glial proliferation (white arrows) in the subcortical area of trauma-induced cerebral hemisphere. HE, x 90 (A. Trauma, B. Trauma+melatonin group)

The number of red neurons in the Melatonin group was significantly lower than that in the Trauma and Trauma plus Placebo groups. The administration of melatonin prevented the formation of ischemia.

Discussion

Red neurons that exhibit homogeneous eosinophilic cytoplasm and, pyknosis, karyorrhexis and karyolysis of the nuclei are known to indicate neurons exposed to ischemia [12]. Therefore, we used the number of red neurons to show the grade of ischemic damage in our study. Based on our results, ischemia began right after trauma and showed gradual increase, and melatonin administration significantly alleviated the formation of cerebral ischemia.

Post-traumatic cerebral ischemic damages are characterized by an imbalance between cerebral oxygen supply and consumption. It was indicated that the major mechanisms causing cerebral ischemic damage are cerebral perfusion impairment, related to second phase arterial vasospasm together with the extravasation of blood due to structural damage in the intracerebral arteries resulting from mechanical trauma, and the increase in focal glucose metabolism [7,13-16].

Melatonin, an endogenous indolamine produced in the pineal gland from an amino acid, tryptophan, is a natural agent that has very beneficial effects. Various studies have shown that melatonin prevents vasospasm in cerebral arteries, reduces ischemic damage after trauma and reduces neuronal damage in cerebral traumas with its protective/preserving effect on neurons [8,9,17,18]).

These data suggested that the protective effect of melatonin on secondary brain injury might be due to its preventive effect on ischemia formation, by stopping the progress of posttraumatic ischemic process.

Limitations

We investigated the histopathological parameters and ischemic findings only. These parameters do not directly demonstrate a link between melatonin's protective and anti-ischemic effects. Therefore, further, larger studies including other parameters are required to reach definite conclusions.

Conclusion

Melatonin, a well-tolerated agent which can overcome the morpho-physiological barriers such as the blood-brain barrier, can be used as a potential therapeutic agent to prevent posttraumatic secondary brain injury by reducing ischemic injury. However, further studies with other parameters are required to demonstrate the direct links between melatonin's protective effect on secondary cerebral injury and preventive effect on ischemia formation.

Acknowledgements

The authors thank Nusret Akpolat for histopathological preparations and analysis.

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